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Morpho-physiological and homeostatic adaptive responses triggered by omeprazole enhance lettuce tolerance to salt stress

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ABSTRACT

Natural or synthetic small molecules (< 500 Da), bioactive at very low concentrations, can potentially increase plant tolerance and resilience to abiotic stresses and improve the resources use efficiency (RUE) of a wide range of crops. Hence, they represent a promising tool in coping with the increasing global food demand imposed by climate change. In this study, the responses of butterhead lettuce (cv. Trocadero) treated with omeprazole (OMP), a benzimidazole inhibitor of animal proton pumps, were studied. OMP was applied as substrate drench at five rates (0, 10, 50, 100 or 200 μM) on lettuce plants grown under nonsaline or saline conditions of 1 or 30 mM NaCl. Increasing NaCl concentration decreased lettuce fresh and dry biomass by 37% and 25% in the 0 μM OMP treatment, respectively; whereas these reductions were mitigated by the 10 μM (12% and 19%, respectively) and 50 μM (15% and 14%, respectively) OMP application. Though OMP was not directly involved in ion homeostasis and K^+/Na^+ ratio regulation, treatment with 10 μM OMP under saline conditions decreased Na^+ in leaves and Cl^- in leaves and roots while increasing NO_3^- concentration in both organs. The synthesis of nitrogenous osmolytes may be implicated in increasing salt tolerance and the sustenance of transpiration and photosynthesis. Under nonsaline conditions, OMP increased root biomass, improving nutrient and water uptake, and therefore RUE.

1. Introduction

Salinity is one of the most widespread threats to sustainable agriculture, particularly severe in arid and semiarid regions, able to jeopardize crop production by causing a direct reduction of global mean yield by up to 20% (Munns and Tester, 2008; Aslam et al., 2017).

Ions such as Na^+, Cl^- and SO_4^{2-} present in irrigation water can accumulate in the soil; while in alkaline soils, characterized by high concentrations of carbonate and bicarbonate, Ca^{2+} and Mg^{2+} ions may precipitate upon evapotranspiration, leaving the soluble Na^+ exceeding the concentration of beneficial nutrients (Rouphael et al., 2017a). Excess Na^+ is able to increase the soil osmotic potential, thereby decreasing the water influx into the plant roots, and interferes with the cation exchange complex of clay particles, causing soil compaction, low porosity and consequently hypoxia (Carillo et al., 2011). Intensive farming, seawater intrusion into freshwater aquifers, use of brackish water for irrigation and decrease in natural precipitation further contribute to soil salinization and adversely impact crop productivity (Annunziata et al., 2017).

Mild salt concentrations in the root zone may not affect crop yield and product quality provided that the critical species-specific concentration is not reached or the plants are not in a sensitive growth phase such as the germination and seedling stages (De Pascale et al., 2012; Ferchichi et al., 2018). However, when salt concentration in the root zone builds up, salt sensitive plants experience initially osmotic stress as an inhibition of water uptake and reduced transpiration rate, with a consequent inhibition of cell division-expansion and arrested plant growth (Maggio et al., 2001; Munns and Tester, 2008; Gorham et al., 2010). Subsequently, if salt stress severity and duration increase and plant exposure to salinity is prolonged or permanent, mature leaves experience first an ionic stress. In particular, Na^+ and Cl^- interfere with K^+ and NO_3^- uptake mechanisms, respectively, causing

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nutritional imbalance. Moreover, Na^+ can substitute K^+ in key enzymatic reactions thereby impairing the plant antioxidant defense system and metabolism (Shabala and Cuin, 2008; Shabala, 2013). These disorders entail dysfunction in fundamental mechanisms like CO_2 assimilation rate, stomatal conductance and transpiration thus decreasing plant viability and crop yield (Yousfi et al., 2010; De Pascale et al., 2012; Rouphael et al., 2017a; Woodrow et al., 2017).

Under salinity, plants can enact ubiquitous mechanisms involving the synthesis and/or accumulation of small molecules (< 500 Da) which directly or indirectly trigger cell osmoregulation, homeostasis and protection against oxidative stress (Kaschani and Van der Hoorn, 2007; Hasegawa, 2013; Lace and Prandi, 2016). As an example, plant hormones like ABA allow plants to acclimate to salinity by closing stomata and accumulating numerous proteins and metabolites for osmotic adjustment (Ryu and Cho, 2015). ABA is directly involved in P5CS gene expression for proline accumulation in Arabidopsis and durum wheat under salinity (Strizhov et al., 1997; Woodrow et al., 2017). Moreover, other small molecules, among which primary or secondary metabolites, like glycine betaine, proline or GABA, can function as osmolytes and antioxidants (Carillo, 2018).

However, exogenous small natural or synthetic bioactive molecules, also derived from human or animal research, may be equally useful in ameliorating toxicity symptoms induced by salinity or in eliciting plant tolerance to salt stress (Kaschani and Van der Hoorn, 2007; Chan and Shi, 2015; Lace and Prandi, 2016). Recently, one such small molecule of 345 Da, omeprazole (OMP), has been shown to confer multiple beneficial effects on tomato plants exposed to salinity (Van Oosten et al., 2017; Rouphael et al., 2018). OMP is a substituted benzimidazole inhibitor of the P-type IIC proton pump H^+/K^+-ATPases in the gastric parietal cell, essential for the treatment of gastric or duodenal ulcer, gastroesophageal reflux, and other hypersecretory states (Fellenius et al., 1981; Puscas et al., 1999). Even though plant ATPases have a very low homology with animal H^+/K^+-ATPase (Sweadner and Donnet, 2001), OMP has been proven effective in increasing plant growth and tolerance to NaCl salinity in tomato when applied at 1–10 μM concentrations over short-term trials (Van Oosten et al., 2017) and up to 100 μM in long-term ones (Rouphael et al., 2018). OMP has a hormone-like activity, deriving from its capacity to modulate plant hormones and their intricate signaling networks. In particular, it is involved in the induction of cutin biosynthesis, alteration of membrane lipids, synthesis and accumulation of osmoprotectants or antioxidants like polyamines, phenolics and carotenoids and in the control of morphological root parameters (Rouphael et al., 2018). All these actions contribute synergistically to ionic homeostasis through decrease of Na^+ and Cl^- concentrations in leaf tissues, improved nutrient acquisition response, photosynthesis and plant performance under salinity (Rouphael et al., 2018). Notwithstanding OMP may enhance stress tolerance by acting on multiple pathways/processes, its molecular targets are not identifiable yet. However, given this small molecule's proven dual function resulting in both growth promotion and stress protection, it can be extremely useful for identifying the key components involved specifically in plant adaptation to salinity (Ruggiero et al., 2004; Van Oosten et al., 2017). Nonetheless, OMP's beneficial effects have been demonstrated on tomato plants which is a species moderately tolerant/sensitive (days to months) to salt-exposure (Bai et al., 2018), yet it is still unclear if these effects are strictly dependent on plant species and/or OMP concentration.

Unlike tomato, low levels of salinity can severely affect both the quality and yield of salt sensitive leafy vegetables, such as lettuce, causing necrotic lesions on leaf margins, reducing head formation and growth, and overall decreasing marketable biomass and quality (Lucini et al., 2015; Rouphael et al., 2016). Thus, it is of great importance to leafy vegetable growers, extension specialists as well as scientists to comprehend how OMP application and dose can trigger adaptation to NaCl stress through morpho-physiological responses and ion homeostasis. Accordingly, in this work five levels of OMP (0, 10, 50, 100 and 200 μM) were applied to lettuce under 1 or 30 mM NaCl salinity conditions in order to analyze the interactive response to OMP concentration × salinity in lettuce. In this way, we were able to assess the ability of this greenhouse leafy vegetable crop to accumulate or exclude beneficial and toxic ions from the plant tissues, to grow and perform leaf CO_2/H_2O exchange under two salinity conditions while treated by different OMP concentrations. The comprehension of OMP action on lettuce could prove particularly useful for the protection and growth promotion of horticultural plants highly sensitive to salinity.

2. Materials and methods

2.1. Plant material and greenhouse conditions

Butterhead lettuce seeds (*Lactuca sativa* L.; Trocadero) were germinated in plates containing vermiculite on 1 June 2016. The Trocadero cultivar is characterized by medium-sized head, bright green color and high resistance to *Bremia*. This cultivar was selected as the most representative commercial variety grown in Italy during the spring-summer cropping season (April–July) under greenhouse conditions. The butterhead lettuce seedlings were transplanted sixteen days after sowing (16 June) at the three-true-leaf stage into plastic pots (diameter 16 cm and height 16 cm) containing a peat/perlite mixture in 2:1 volume ratio. The plastic pots were placed on aluminum troughs (1.8 × 7 m) at a plant density of 16 plants m^{-2}. Butterhead lettuce plants were grown under natural light conditions. The trial was carried out under glasshouse conditions at the experimental station of the Department of Agricultural Sciences, University of Naples Federico II, located in Bellizzi, southern Italy (43° 31″ N, 14° 58″ E; 60 m above sea level). Inside the glasshouse, the daily air temperature varied between 18 and 32 °C and the relative humidity was 55%/70% during day/night, respectively.

2.2. Experimental design, omeprazole and NaCl application and nutrient solution management

The glasshouse experiment was performed as a two-way factorial design encompassing combinations of two NaCl concentrations in the nutrient solution (non salt control 1 or 30 mM) and five omeprazole (OMP) application levels (0, 10, 50, 100 or 200 μM). There were three replicate plots per treatment arranged in a completely randomized design, amounting to a total of thirty experimental units with six plants each.

The OMP was applied four times during the growing cycle at weekly intervals as substrate drench at a uniform rate of 100 ml per lettuce plant. The applications were initiated eight days after transplanting (DAT; 23 June).

The basic nutrient solution was a modified Hoagland and Arnon formulation. All chemicals used were of analytical grade. The composition of the nutrient solution was: 8.0 mM N–NO_3^-, 1.0 mM P, 1.0 mM S, 3.0 mM K, 3.0 mM Ca, 0.7 mM Mg, 1 mM NH_4^+, 1 mM Na, 1 mM Cl, 25 μM Fe, 10 μM Mn, 0.3 μM Cu, 1.8 μM Zn, 25 μM B, and 0.3 μM Mo, with an electrical conductivity (EC) of 1.4 dS m^{-1}. The saline nutrient solutions had the same basic composition plus an additional 29 mM of NaCl, giving EC values of 4.5 dS m^{-1}. The pH of the nutrient solution in nonsaline and saline treatments was 5.9 ± 0.2. One auto-compensating dripper per plant having a flow rate of 2 L h^{-1} was used to ensure uniform irrigation, supply of nutrient solution and stress induction. The frequency of irrigation varied from two to five fertigation cycles of 1–3 min per day. Irrigation volume was maintained at 20% above the volume required to restore field capacity, i.e., leaching was monitored at 20% fraction (Colla et al., 2012). Leaching fractions were adopted to maintain the EC in the substrate at the targeted level (Colla et al., 2012, 2013). Saline treatments were initialized in concomitance with OMP application, 8 DAT.

2.3. Yield, biomass production, growth measurements and leaf color measurements

At the end of the experiment (20 July; 35 DAT) the number of leaves per plant was recorded. The total leaf area per plant was measured using an electronic area meter (Li-Cor3000, Li-Cor, Lincoln, NE, USA). All plants of lettuce per experimental plot were harvested and separated in leaves and roots. Both leaf and root tissues were dried at 70 °C for 72 h until they reached a constant weight, recorded on an analytical balance, which corresponded to their dry biomasses. Root-to-shoot ratio was also calculated. The dried leaf and root tissues were used for mineral composition analysis.

Before harvest, leaf color was measured on the upper part of eight butterhead lettuce leaves per experimental unit plot, using an 8 mm-aperture Minolta CR-300 Chroma Meter (Minolta Camera Co. Ltd, Osaka, Japan). The Chroma meter was calibrated with a Minolta standard white plate. The *Commission internationale de l'éclairage* (CIE) color space parameters were also recorded in the present study.

2.4. Relative water content, leaf water potential and leaf gas exchange

At the end of the experiment (35 DAT) water status parameters of butterhead lettuce plants were evaluated. A dew-point psychometer (WP4; Decagon Devices, Pullman, WA) was used to measure leaf water potential. The Relative Water Content (RWC) was measured according to the following formula: $RWC = [FW - DW]/[TW - DW] \times 100$; where FW, DW and TW are the fresh, dry and turgid weight, respectively.

Two times during the growing cycle (11 and 20 July, corresponding to 26 and 35 DAT) the net CO_2 assimilation rate (P_n), stomatal conductance (g_s) and transpiration rate (E) were measured with a portable gas exchange analyzer (LCA-4; ADC BioScientific Ltd., Hoddesdon, UK) equipped with a broadleaf chamber (cuvette window area, 6.25 cm^2). This measurement was carried out within 2 h across solar noon (i.e. between 11.00 and 13.00) on six fully expanded leaves per treatment. Photosynthetically Active Radiation (PAR), Relative humidity (RH) and CO_2 concentration (660 ± 100 and 500 ± 100 µmol m^{-2} s^{-1}, RH 42 ± 3 and 40 ± 3%, and 360 ± 2 and 368 ± 2 ppm, respectively) were set at ambient value and the air flow rate was 400 ml s^{-1}.The intrinsic Water Use Efficiency (WUE_{int}) was calculated as Pn/g_s.

2.5. Ion measurements and partitioning

Dried leaf and root tissues ground in a Wiley Mill to pass through an 841 µm screen were used for mineral profile analysis. The anions (NO_3^-, PO_4^{3-}, SO_4^{2-} and Cl^-) and cations (K^+, Ca^{2+}, Mg^{2+} and Na^+) in leaf and root tissues were separated and quantified by ion chromatography (ICS-3000, Dionex, Sunnyvale, CA, USA) coupled to a conductivity detector, using an IonPac CG12 A guard column and IonPac analytical column for cations, as well as an IonPac AG11-HC guard column and IonPac AS11-HC analytical column for anions (Rouphael et al., 2017b).

2.6. Statistical analysis, principal component analysis and heat map analysis

Analysis of variance (two-way ANOVA) of the morpho-physiological and mineral composition experimental data was performed using IBM SPSS Statistics 20 for Windows (IBM SPSS Inc., USA). To separate treatment means within each measured parameter, Duncan's Multiple Range Test was performed at $P \leq 0.05$. Principal Component Analysis (PCA) was also conducted using Minitab 16.2.1 statistical software, aimed to extract trends when multiple qualitative variables were used by formulating new variables correlated to the original ones (Cardarelli et al., 2017; Kyriacou et al., 2019). The PCA outputs included variable loading and also treatment component scores to each selected

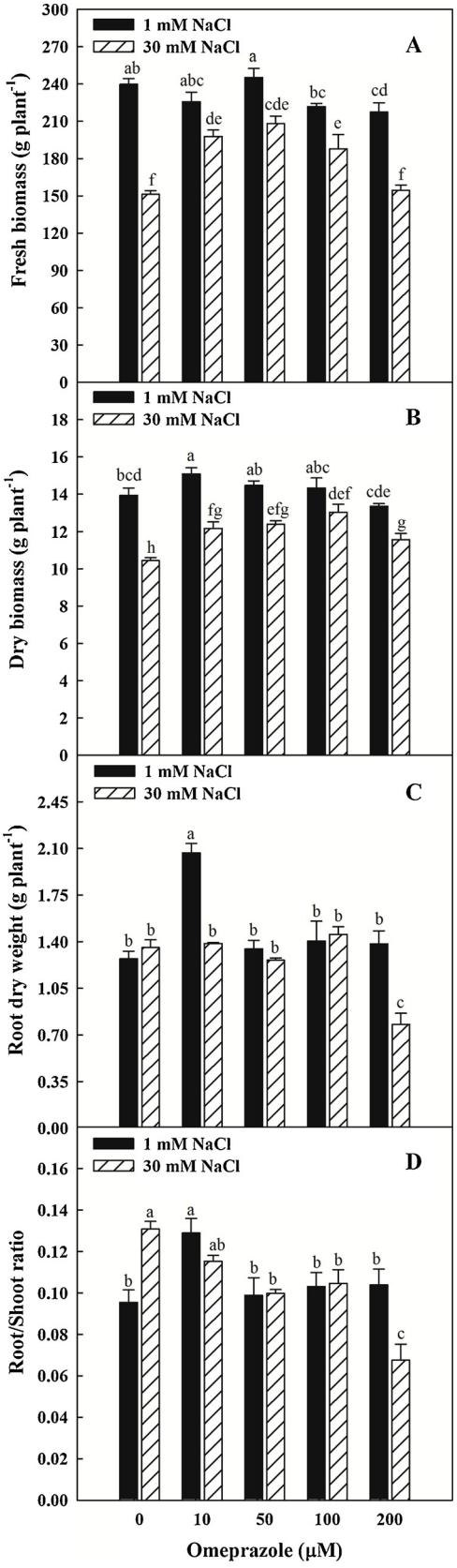

Fig. 1. Effects of NaCl concentration in the nutrient solution and omeprazole application rates on fresh yield (A), dry biomass (B), root dry weight (C) and root-to-shoot ratio of greenhouse lettuce plants. Different letters indicate significant differences according to Duncan's test (P = 0.05). Values are the means of four replicate samples. Vertical bars indicate ± SE of means.

Table 1
Analysis of variance and mean comparisons for leaf number, leaf area, leaf dry matter content and CIE colour parameters L* (brightness), a* (+a*= red; −a* = green) and b* (+b* = yellow; −b*= blue) of lettuce plants grown under two salinity levels and treated with omeprazole (OMP) at five rates of application.

Source of variance	Leaf number (no. plant^{-1})	Leaf area (cm^2 plant^{-1})	Leaf dry matter (%)	L*	a*	b*
Salinity (mM NaCl) (S)						
1	41.5 a	3536 a	5.95	47.50 a	−18.94	29.58 a
30	39.0 b	3086 b	6.51	45.24 b	−18.48	27.47 b
Omeprazole (μM) (OMP)						
0	38.1 b	3067 c	6.40	45.63	−18.44	28.23
10	42.0 a	3452 a	6.06	46.29	−18.88	29.10
50	41.4 a	3507 a	6.06	47.07	−18.93	29.19
100	40.7 a	3373 ab	6.24	47.21	−19.06	29.13
200	38.9 b	3157 bc	6.39	45.66	−18.26	26.96
Significance						
Salinity (mM NaCl) (S)	***	***	***	*	ns	**
Omeprazole (μM) (OMP)	***	***	ns	ns	ns	ns
S × OMP	ns	ns	**	ns	ns	ns

ns,*,**, *** Nonsignificant or significant at P ≤ 0.05, 0.01, and 0.001, respectively. Different letters within each column indicate significant differences according to Duncan's multiple-range test (P = 0.05).

component. A heat map analysis summarizing the plant responses to OMP × salinity treatments was generated using the https://biit.cs.ut.ee/clustvis/ online program package with Euclidean distance as the similarity measure and hierarchical clustering with complete linkage. Data were visualized using a false color scale, with red indicating an increase and blue a decrease of values (Rouphael et al., 2018).

3. Results

3.1. Fresh yield, growth response and leaf colorimetric attributes

Our morphological data clearly indicated that OMP triggered two specific effects: (i) improved fresh and dry biomass and (ii) increased tolerance of butterhead lettuce under NaCl stress (Fig. 1 and Table 1). Specifically, for crop productivity and biomass production we observed that OMP under both nonsaline and saline conditions had a dose dependent effect (Fig. 1). For instance, in both untreated and OMP-treated lettuce plants, fresh yield and dry biomass decreased in response to an increase in nutrient solution salinity from 1 to 30 mM NaCl (Fig. 1). However, the fresh biomass differential between high salinity (30 mM NaCl) and low salinity (1 mM NaCl) was clearly lower in plants treated with 10 μM OMP (12% reduction), 50 μM OMP (15% reduction) and 100 μM OMP (15% reduction) compared to the untreated plants (37% reduction) and the 200 μM OMP (29% reduction) treatment. A similar effect was observed for the dry biomass, wherein the high-low salinity differential was lower in all OMP treatments (10–200 μM mean reduction = 14%) compared to the untreated plants (25% reduction). Moreover, under non-saline conditions (1 mM NaCl), the drench application of OMP elicited a dose-specific increase of root dry weight and the R/S ratio manifested at 10 μM but not between the dose range of 50–200 μM, whereas under saline conditions (30 mM NaCl) the lowest root dry weight values were recorded when OMP was applied at 200 μM (Fig. 1). Similarly, the lowest R/S ratio under saline conditions was observed in lettuce treated with 200 μM OMP (Fig. 1).

Irrespective of OMP application (salinity × OMP interaction = ns), the number of leaves and total leaf area of butterhead lettuce declined under increased nutrient solution salinity (Table 1). Averaged over salt treatment levels, significant differentiation in terms of leaf number and area was observed in response to OMP application, with the higher values recorded at 10, 50 and 100 μM OMP as opposed to 0 and 200 μM OMP. Moreover, leaf dry matter percentage was significantly affected by salinity × OMP interaction with the highest values recorded in untreated lettuce plants grown under saline conditions (data not shown). Leaf green color intensity (expressed by negative a* values) was not affected by either salinity or OMP, whereas lightness (L*) and yellow color intensity (expressed by positive b* values) were influenced solely by salt treatment (Fig. 1). Specifically, increasing the nutrient solution salinity from 1 to 30 mM NaCl yielded darker lettuce leaves, expressed by decreasing L* values, and also less yellow, denoted by lower b* values (Table 1).

3.2. Physiological response

At 26 DAT, the 30 mM NaCl treatment resulted in lower net CO_2 assimilation rate (A_{CO2}) compared to the 1 mM NaCl treatment over all OMP substrate drench application rates except at 10 μM OMP, which resulted in A_{CO2} not different from that observed under 1 mM NaCl (Table 2). Moreover, when averaged over both NaCl concentrations, the A_{CO2} at 35 DAT responded to the drench application of OMP in a dose–dependent manner with peak values observed at 10 and 50 μM and the lowest values observed at 0 and 200 μM OMP (Table 2). The peak A_{CO2} observed at 35 DAT in 10 and 50 μM OMP-treated lettuce plants was accompanied by lower stomatal resistance (Table 2).

Irrespective of salt-treatment levels, the transpiration rate (E) at 35 DAT of lettuce plants treated with 10, 50 and 100 μM was significantly higher compared to 0 μM control and 200 μM OMP (Table 2). Conversely to the leaf gas exchange parameters, no significant differences between treatments were recorded in RWC of lettuce leaves (Table 2). Moreover, the mean leaf water potential (LWP) of all OMP treatments decreased by 33.3% when the nutrient salinity level increased from 1 to 30 mM NaCl, whereas the LWP in 200 μM OMP treated-plants was lower than that of plants receiving 10 or 100 μM OMP substrate drench (Table 2). Finally, a strong positive correlation was found between the levels of E and A_{CO2} ($r = 0.72$; $P < 0.01$) and between A_{CO2} and fresh yield ($r = 0.92$; $P < 0.001$), independently of salinity and OMP application.

3.3. Ion profile and partitioning

Except for the bivalent Mg^{2+} cation in the roots and the toxic Cl^- anion in both leaves and roots, which incurred significant salinity × OMP interaction, all the macronutrients (NO_3^-, PO_4^{3-}, K^+ and Ca^{2+}) and Na^+ in both leaves and roots were mainly influenced by salinity and to a lesser degree by the drench application of OMP (Table 3). The NO_3^-, PO_4^{3-}, K^+, Ca^{2+} and Mg^{2+} concentrations in both leaves and roots were negatively affected by NaCl concentration in the nutrient solution. Specifically, increasing the NaCl concentration from 1 to 30 mM decreased the leaf and root concentrations of NO_3^- (by 35.5% and 30.9%, respectively), PO_4^{3-} (by 11.4% and 16.2%, respectively), K^+ (by 19.7% and 14.3%, respectively), Ca^{2+} (by 51.8%

Table 2
Analysis of variance and mean comparisons for net CO_2 assimilation rate (A_{CO2}), stomatal resistance (r_s), transpiration rate (E), intrinsic Water Use Efficiency (WUE), relative water content (RWC) and leaf water potential (LWP) of lettuce plants grown under two salinity levels and treated with omeprazole (OMP) at five rates of application.

Source of variance	A_{CO2}		r_s		E		WUEi		RWC	LWP
	(μmol CO_2 m^{-2} s^{-1})		(m^2 s^1 mol^{-1})		(mol H_2O m^{-2} s^{-1})		(μmol CO_2 mol^{-1} H_2O)		(%)	(MPa)
	26 DAT	35 DAT	26 DAT	35 DAT	26 DAT	35 DAT	26 DAT	35 DAT		
Salinity (mM NaCl) (S)										
1	13.59	10.61 a	2.58	4.22	4.33	3.77 b	3.17	2.87 a	79.36	−0.76 a
30	11.40	8.79 b	3.03	3.78	3.87	4.19 a	2.98	2.12 b	76.73	−1.14 b
Omeprazole (μM) (OMP)										
0	11.67	7.79 c	3.31	5.07 a	3.91	3.40 b	3.00	2.49	81.22	−1.06 ab
10	13.41	11.09 a	2.36	3.24 b	4.33	4.48 a	3.13	2.51	76.65	−0.80 a
50	12.56	11.97 a	2.65	2.54 b	4.12	4.51 a	3.07	2.67	78.17	−0.99 ab
100	12.53	10.07 b	2.99	3.66 ab	4.02	4.19 a	3.18	2.49	75.84	−0.80 a
200	12.31	7.59 c	2.72	5.48 a	4.10	3.33 b	3.00	2.30	78.33	−1.09 b
S × OMP										
1 mM NaCl × 0 μM OMP	13.36 a	8.87	3.01	5.86	4.32 abc	2.96	3.10	3.21	82.28	−0.77
1 mM NaCl × 10 μM OMP	13.26 a	11.79	3.14	3.78	4.08 abcd	4.25	3.28	2.82	75.39	−0.80
1 mM NaCl × 50 μM OMP	13.48 a	13.44	1.83	2.31	4.52 ab	4.54	3.00	2.97	79.62	−0.80
1 mM NaCl × 100 μM OMP	14.02 a	10.86	2.62	4.95	4.30 abc	3.65	3.31	3.01	80.11	−0.60
1 mM NaCl × 200 μM OMP	13.86 a	8.11	2.29	4.21	4.41 abc	3.46	3.15	2.36	79.38	−0.82
30 mM NaCl × 0 μM OMP	9.98 c	6.71	3.61	4.28	3.51 d	3.85	2.90	1.78	80.16	−1.36
30 mM NaCl × 10 μM OMP	13.55 a	10.39	1.58	2.71	4.59 a	4.71	2.97	2.21	78.53	−0.80
30 mM NaCl × 50 μM OMP	11.65 b	10.50	3.47	2.77	3.72 cd	4.48	3.14	2.37	76.72	−1.17
30 mM NaCl × 100 μM OMP	11.05 bc	9.28	3.35	2.37	3.73 bcd	4.74	3.06	1.97	71.58	−1.01
30 mM NaCl × 200 μM OMP	10.76 bc	7.07	3.15	6.75	3.79 bcd	3.20	2.85	2.24	77.28	−1.37
Significance										
Salinity (mM NaCl) (S)	***	***	ns	ns	**	**	ns	***	ns	***
Omeprazole (μM) (OMP)	*	***	ns	*	ns	***	ns	ns	ns	*
S × OMP	**	ns	ns	ns	*	ns	ns	ns	ns	ns

ns,*,**, *** Nonsignificant or significant at $P \leq 0.05$, 0.01, and 0.001, respectively. Different letters within each column indicate significant differences according to Duncan's multiple-range test ($P = 0.05$).

and 31.7%, respectively) and Mg^{2+} (by 40.3% and 49.9%, respectively); whereas an opposite trend was observed for the toxic Na^+ and Cl^- ions (Table 3). Sodium and chloride concentrations in both leaves and roots spiked with increasing nutrient solution salinity, the concentrations of both toxic ions being highest in the roots. The K^+/Na^+ ratio, initially equal to 18.9 in leaves and 2.3 in roots, was drastically reduced by the 30 mM NaCl treatment to 1.8 and 0.8, respectively.

The substrate drench application of OMP, averaged over salinity levels, induced significant increase of NO_3^-, K^+ and Ca^{2+} concentrations in leaf tissues which were higher on average by 14.0%, 8.6% and 17.0%, respectively, at the lower OMP doses (10 and 50 μM) compared to the 200 μM OMP and the control (Table 3). The beneficial application of OMP on root nutritional status was observed on nitrate and potassium concentrations in both leaves and roots, and also in leaf calcium concentrations (Table 3). Interestingly, significant OMP × salinity interaction was observed as the OMP treatment effectively reduced the Cl^- accumulation in both leaves and roots under salt stress conditions (30 mM NaCl) but not under nonsaline (1 mM NaCl) conditions. Under saline conditions the OMP application significantly reduced Cl^- accumulation in leaf tissue in a dose-dependent manner: −26.8% and −13.0% in response to 10 and 50 μM OMP, respectively. Finally, root Cl^- concentration was lowered by 26.3%, when 10, 50, 100 or 200 μM OMP was delivered to lettuce plants, with no significant difference between the four OMP treatments (Table 3).

3.4. Heat map analysis

A heat map representing the changes in the parameters analyzed in response to the salinity × OMP treatments provided an integrated view of their effects on lettuce plants (Fig. 2). The heat-map identified two main clusters corresponding to 1 and 30 mM NaCl. This indicated that salinity was the main clustering factor responsible for different effects in dependence to its own concentration (i.e., 1 or 30 mM NaCl). Indeed, two separated sub-clusters could be defined under each main cluster, which illustrate the salinity × OMP interaction. In particular, the 10 μM OMP × 1 mM NaCl subcluster separated from all other nonsaline treatments because of its higher root dry weight, R/S ratio, as well as high Mg^{2+} concentration in the roots (Fig. 2). On the other hand, the 10 μM OMP × 30 mM NaCl treatment was distanced from all other saline treatments mainly because of its lower r_s, and higher total leaf area, A_{CO2} and LWP. In general, the nonsaline treatments were characterized by lower toxic ion concentrations in both leaves and roots, better crop performance (higher fresh and dry biomass) and nutritional status (higher macronutrient concentration) in both plant tissues, and were able to maintain higher photosynthetic activity (Fig. 2).

3.5. Principal component analysis

A comprehensive view of the morpho-physiological and mineral composition and partitioning of greenhouse lettuce plants in response to OMP application under both nonsaline and saline conditions was obtained through principal component analysis (PCA). The first two principal components (PCs) were associated with Eigen values higher than 1 and explained 76.8% of the cumulative variance, with PC1 accounting for 67.4% and PC2 for 9.3% (Fig. 3). PC1 was positively correlated to leaf Ca^{2+}, K^+ and PO_4^{3-} concentration, NO_3^- and Mg^{2+} concentration both in leaf and root tissues, dry biomass, fresh yield, A_{CO2}, as well as leaf number and area. PC1 was also negatively correlated to leaf Na^+ and Cl- concentration in both plant tissues, as well as to stomatal resistance. Moreover, PC2 was positively correlated to root dry weight and R/S ratio, and negatively correlated to RWC, transpiration and root calcium concentration (Fig. 3).

The interpretive usefulness of PCA in preharvest and postharvest studies on multiple biochemical, physiological, ion homeostasis and quality traits has been reported previously in a series of scientific studies (Kyriacou et al., 2016; Carillo, 2018; Rouphael et al., 2019). This

Table 3
Analysis of variance and mean comparisons for nitrate, phosphate, potassium, calcium, magnesium, sodium and chloride ions in leaves and roots of lettuce plants grown under two salinity levels and treated with omeprazole (OMP) at five rates of application.

Source of variance	NO_3^- (mg g^{-1} DW)		PO_4^{3-} (mg g^{-1} DW)		K^+ (mg g^{-1} DW)		Ca^{2+} (mg g^{-1} DW)		Mg^{2+} (mg g^{-1} DW)		Na^+ (mg g^{-1} DW)		Cl^- (mg g^{-1} DW)	
	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots
Salinity (mM NaCl) (S)														
1	60.18 a	69.27 a	14.33 a	27.44 a	47.88 a	33.65 a	12.44 a	3.19 a	3.65 a	2.19	2.53 b	14.83 b	5.03	1.77
30	38.80 b	47.86 b	12.69 b	22.98 b	38.46 b	28.70 b	5.98 b	2.18 b	2.18 b	1.11	21.37 a	31.95 a	34.11	37.75
Omeprazole (μM) (OMP)														
0	46.87 b	48.17 b	14.07	27.79	41.53 ab	27.05 b	8.60 b	2.94	2.94	1.47	13.55	23.64	21.96	25.10
10	52.82 a	62.84 a	13.79	25.52	44.88 a	32.78 a	10.02 a	2.40	2.95	1.98	9.61	22.07	16.97	18.49
50	52.87 a	63.43 a	13.22	22.73	44.37 a	29.61 ab	9.84 a	2.51	3.13	1.74	12.13	24.01	19.78	19.06
100	49.01 ab	61.38 a	13.93	25.19	44.60 a	32.95 a	9.22 ab	2.75	2.92	1.53	12.13	22.14	18.96	17.96
200	45.88 b	57.02 ab	12.53	24.81	40.45 b	33.49 a	8.39 b	2.82	2.64	1.53	12.30	25.09	20.17	18.18
S x OMP														
1 mM NaCl × 0 μM OMP	58.00	57.73	15.61	31.58	45.68	29.81	11.56	3.75	3.51	1.76 bcde	2.30	14.34	4.40 d	2.39 c
1 mM NaCl × 10 μM OMP	63.25	70.34	14.00	27.71	49.98	34.35	13.34	2.89	3.68	2.97 a	2.19	13.53	5.05 d	1.96 c
1 mM NaCl × x 50 μM OMP	66.61	79.58	14.11	23.18	50.22	31.92	13.33	2.93	4.07	2.42 ab	3.31	12.85	5.18 d	1.33 c
1 mM NaCl × 100 μM OMP	58.04	73.70	14.37	28.60	48.41	36.68	12.49	3.33	3.62	2.00 bc	2.51	14.58	5.21 d	1.67 c
1 mM NaCl × x 200 μM OMP	54.98	65.02	13.54	26.13	45.10	35.48	11.48	3.06	3.37	1.81 bcd	2.33	18.85	5.32 d	1.48 c
30 mM NaCl × x 0 μM OMP	35.74	38.60	12.52	23.99	37.38	24.29	5.64	2.12	2.38	1.18 de	24.81	32.94	39.52 a	47.81 a
30 mM NaCl × 10 μM OMP	42.38	55.34	13.59	23.33	39.79	31.21	6.69	1.91	2.21	0.99 e	17.03	30.62	28.89 c	35.02 b
30 mM NaCl × 50 μM OMP	39.13	47.28	12.32	22.29	38.52	27.29	6.34	2.10	2.20	1.05 de	20.95	35.18	34.38 b	36.79 b
30 mM NaCl × x 100 μM OMP	39.98	49.06	13.50	21.78	40.80	29.22	5.94	2.18	2.23	1.06 de	21.75	29.71	32.71 ab	34.25 b
30 mM NaCl × 200 μM OMP	36.78	49.02	11.52	23.49	35.81	31.49	5.30	2.58	1.91	1.25 cde	22.28	31.33	35.03 b	34.87 b
Significance														
Salinity (mM NaCl) (S)	***	***	**	**	***	***	***	***	***	***	***	***	***	***
Omeprazole (μM) (OMP)	*	*	ns	ns	*	**	*	ns	ns	ns	ns	ns	ns	**
S × OMP	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	ns	*	*

ns,*,**, *** Nonsignificant or significant at $P \leq 0.05$, 0.01, and 0.001, respectively. Different letters within each column indicate significant differences according to Duncan's multiple-range test ($P = 0.05$).

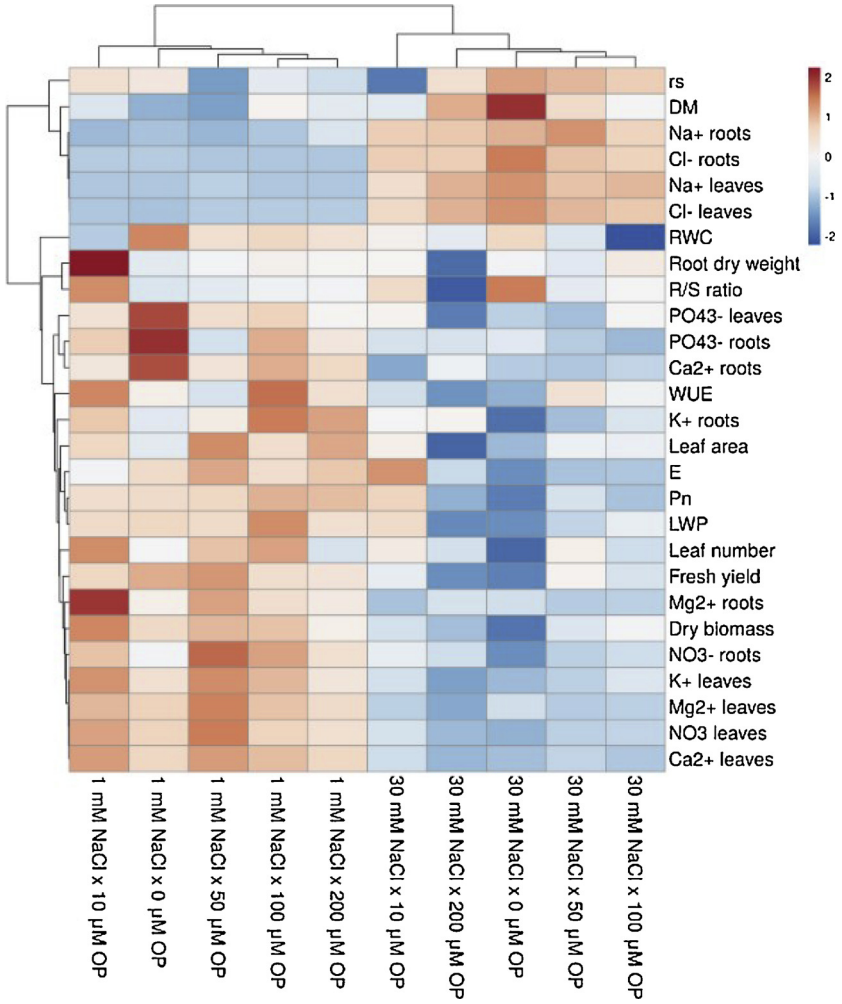

Fig. 2. Cluster heat map analysis summarizing plant responses to OMP and salinity treatments. It was generated using the https://biit.cs.ut.ee/clustvis/ online program package with Euclidean distance as the similarity measure and hierarchical clustering with complete linkage.

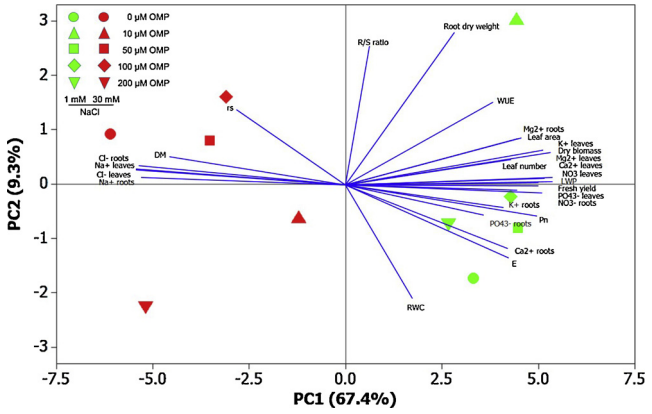

Fig. 3. Principal component loading plot and scores of principal component analysis (PCA) on morphological, physiological parameters and ion concentrations of butterhead lettuce grown under nonsaline or saline nutrient solution (1 or 30 mM NaCl, respectively), and OMP application delivered at five rates (0, 10, 50, 100 or 200 µM).

was the case in the current experiment, since the score plot of the PCA superimposed on the above matrix of variables revealed strong clustering of the two nutrient solution concentrations along PC1, with nonsaline treatment (1 mM NaCl) concentrating fresh yield, dry biomass, plant growth parameters, mineral composition in both leaves and roots, photosynthetic activity, while the saline treatment (30 mM NaCl) stands out for toxic ions (Na^+ and Cl^-), leaf dry matter percentage and stomatal resistance (Fig. 3). The OMP applications were not univocally clustered in respect to PC2, but they changed in dependence to OMP concentration × salinity treatment. Particularly, the 10 µM OMP × 1 mM NaCl was positioned on the positive side of PC1 in the upper quadrant of the PCA score plot, and represented lettuce plants with high shoot and root biomass, better nutritional status and higher WUE_i (Fig. 3). Lettuce plants receiving 0 or 200 µM and especially the 50 or 100 µM OMP were positioned in the lower right quadrant, characterized overall by improved photosynthetic performance and high K^+ and PO_4^{3-} concentrations in roots (Fig. 3). Finally, treatments in the upper left quadrant (50, 100 and especially 0 µM OMP at 30 mM NaCl) were characterized by high concentrations of toxic ions in both plant tissues that consequently affected negatively plant growth and productivity (Fig. 3).

4. Discussion

Salinity affects vegetable crops posing a serious threat to their potential growth and development (Shabala and Munns, 2017; Ferchichi et al., 2018). However, the occurrence and combinatorial effects of salt concentration, time of exposure, cultural environment and sensitivity of species and/or cultivars may dictate remarkable fluctuations in the biomass production and quality of vegetable crops (Colla et al., 2010). Particularly in salt sensitive leafy vegetables, even low levels of salinity

can seriously damage the plants and suppress their fresh biomass and marketable yield (Rouphael et al., 2016, 2017c; Rouphael and Kyriacou, 2018). This was demonstrated in the present experiment on butterhead lettuce plants supplied with 30 mM NaCl. Significant decrease in morphological and physiological traits was noted on NaCl-treated plants; leaf number and leaf area per plant in particular decreased with increasing soil salinity which, in turn, reduced the average lettuce fresh and dry biomass (Ünlükara et al., 2008). Salinity affected transpiration (E), strongly reducing CO_2 assimilation rate (A_{CO2}) and therefore compromising fresh yield. These parameters appeared strongly correlated in butterhead lettuce as previously demonstrated in other species (Katerji et al., 1998).

Photosynthesis, together with cell growth, is one of the primary processes affected by salinity (Munns et al., 2006). This can be the direct derivative of effects, such as lower CO_2 availability due to increased stomatal resistance (r_s), presently exemplified in butterhead lettuce, and the consequent alterations of photosynthetic metabolism; or indirect effects due to the generation of Reactive Oxygen Species (ROS) which can irreversibly damage the photosynthetic machinery and function (Chaves et al., 2009).

The negative effects of salinity were partly mitigated by the application of OMP, a benzimidazole derivative exerting an inhibitory action on animal proton pumps, which improved various butterhead lettuce growth parameters, including leaf number, leaf area, shoot fresh and dry biomass, as previously reported in Van Oosten et al. (2017), as well as photosynthesis. In particular, the enlargement of leaf area induced by OMP independent of salinity indicates that this small molecule is able to exert a protective action owing to which treated plants have no need to minimize water loss even at low r_s, since they can successfully osmoregulate cytosol and vacuole while maintaining adequate levels of net CO_2 assimilation rate (A_{CO2}) (Van Oosten et al., 2017; Carillo et al., 2019). It is noteworthy that the positive effects of OMP on A_{CO2} and LWP, as well as on ion uptake control, were concentration-dependent, as they were particularly pronounced at 10 µM OMP concentration while at 200 µM OMP an inhibitory effect or no effect was observed. These inhibitory effects on butterhead lettuce under saline conditions concern, in particular, the decrease of root growth, R/S ratio, PO_4^{3-} and leaf area, while other parameters remained unaffected. A possible explanation for these effects is that at high concentrations OMP could disturb pH homeostasis and cause an accumulation of ROS with consequent cytotoxic effects, as previously demonstrated in the non-marine microalga *Tetraselmis suecica* (Seoane et al., 2017).

Butterhead lettuce plants, differently than *L. sativa* var. *crispa* (Ünlükara et al., 2008), did not display traits of salt stress tolerance such as, the ability for retaining lower content of Na^+ and Cl^- in leaves than in roots under salinity, or for maintaining high K^+ to Na^+ ratios. In fact, in butterhead lettuce leaves Na^+ and Cl^- increased by tenfold when salinity was increased from 1 to 30 mM NaCl, and K^+ to Na^+ ratios decreased of 90% on average, despite the decline in potassium in the leaves amounted to only 20%. However, when plants were treated with 10 µM OMP, as mentioned above, the concentration of these toxic ions decreased in the leaves, while Cl^- also decreased in the roots. This demonstrated that this small molecule is able to activate a mechanism that increases salt tolerance (Rodrıguez et al., 2005), even though it is not directly involved in ion homeostasis, in particular K^+ to Na^+ ratio regulation, as previously shown by Rouphael et al. (2018).

Since Cl^- competes for NO_3^- transporter proteins affecting NO_3^- uptake and transport and reduces the loading of NO_3^- into the root xylem (Carillo et al., 2005), the decrease of Cl^- in plants treated with OMP could be probably responsible for the simultaneous enhancement of NO_3^- concentration both in roots and leaves. The presence of high levels of NO_3^- implied the maintenance of an active nitrate reductase activity and nitrogen metabolism in roots and shoot, which, in turn, could allow fulfilling the demand for nitrogen-containing compounds such as amino acids and amines, highly contributing to boosting butterhead lettuce performance. Notwithstanding the lower levels of K^+, even a slight increase in N-containing osmolytes, such as proline and glycine betaine which are accumulated in the cytosol, could be sufficient to balance osmotically lettuce leaf cells against the toxic ions Na^+ and Cl^-, compartmentalized in the vacuoles and/or vacuoles plus apoplast (Ferchichi et al., 2018). This relies on the fact that the percentage of cell volume occupied by the cytosol in lettuce leaves, as in spinach, is lower than 4%, while that of vacuoles and/or vacuoles plus apoplast amount to about 85 and 92% of cell volume, respectively (Winter et al., 1994). Besides, several studies show that the oxidative damage occurring under salinity is due to the deregulated production of ROS and the alteration of protective antioxidant mechanisms (Leyva et al., 2011; Hossain et al., 2017). However, the same N-containing compounds involved in the osmoregulation of cytosol, e.g. proline and glycine betaine, in addition to their primary role in osmotically balancing the cytosol, they can strongly contribute to the protection of subcellular structure, buffer redox potential and scavenge ROS under salinity, thus preserving important processes like protein synthesis, enzyme activity and photosynthesis (Annunziata et al., 2017; Shabala and Munns, 2017; Carillo, 2018).

A stimulator effect of OMP was also evidenced on nonsaline 1 mM NaCl plants when treated with 10 µM OMP, particularly in terms of root dry weight and R/S ratio. This beneficial effect on root growth was previously evidenced in tomato by Rouphael et al. (2018), which suggested it could be due to a direct stimulation of root dry biomass, surface and length by OMP-induced endogenous phytohormones, like auxin. This expansion of root biomass and therefore of root volume could allow for better and deeper exploration of soil resources and in particular of water and nitrate before they are lost from the rhizosphere (Tuberosa et al., 2002; Lynch, 2013). In fact, nitrate is particularly prone to leaching because it is highly soluble in water, and therefore it may be easily lost from the root zone (Dathe et al., 2016). Under OMP treatment, the ability of plants to uptake and accumulate NO_3^- and K^+ increased in both root and shoot, while Ca^{2+} increased only in the leaves. Consequently, photosynthetic and transpiration capacity increased along with leaf area and number without affecting WUE, notwithstanding the decrease in r_s.

5. Conclusions

Climate change and environmental abiotic stresses, in particular salinity, significantly threaten crop production and food security worldwide. Thus, new approaches and strategies are required for meeting the growing demand for food and feed, by improving crop resilience and potentially by increasing crop yields in the coming decades. Together with breeding methods and new biotechnological advances, plant scientists have been recently exploring the potential role of small natural or synthetic bioactive molecules, like OMP, with the aim to increase stress tolerance and/or RUE. OMP shows a dose-dependent two-way action on butterhead lettuce. Under control conditions, it improves the plant performance by increasing root biomass and volume, consequently improving uptake of nutrients and water, and therefore enhancing RUE. Under salinity, OMP increases the tolerance of plant to NaCl salt stress probably by altering different biochemical/metabolic processes, which ultimately improve plant capacity for counteracting the detrimental effects of salinity. The primary putative mechanism(s) through which OMP exerts its function is that of preserving nutrient availability, mitigating competitive uptake and transporting or partitioning of toxic ions and beneficial ones. Even if OMP does not appear strictly involved in the control of K^+ to Na^+ ratio, it is able to decrease leaf Na^+ concentration and leaf and root Cl^- concentrations under salinity stress, thereby improving NO_3^- uptake and transport to the leaves and subsequently nitrogen metabolism. In this way, it is ensured the synthesis of protective nitrogenous compounds, instrumental for maintaining osmotic balance, pH stat, ROS control and prevention of physiological inactivation of important enzymatic mechanisms and processes such as transpiration and photosynthesis. Our

studies further confirm the significant benefits deriving by the use of OMP which amount to improving yields of horticultural products, independently of salinity. Although at present it is not possible to hypothesize a large-scale use of this small molecule due to its prohibitive cost, further studies are warranted to unravel the molecular mechanisms involved in its actions and possibly to identify low-cost analogs for wider application.

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